On the Origin of Species: The Madeira River as a Barrier for Small Mammals in the Amazon Basin

Honors Research Thesis

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Abstract

Barriers to dispersal (and hence gene flow between populations) represent an important causal factor promoting speciation. The Amazon River and its major tributaries have been the focus of numerous biogeographical studies because of their potential role in isolating populations. One New World marsupial, the woolly mouse opossum [*Marmosa (Micoureus) demerarae*], currently occupies the entire Amazon Basin, but recent studies have identified distinct, highly-divergent phylogroups across the region. In fact, this taxon may represent several cryptic species, but the potential role of rivers for isolating populations is unknown. The aim of this study was to investigate both morphological and genetic variation among *M. demerarae* populations that straddle the Madeira river, the largest Amazonian tributary. We examined biogeographical patterns of morphology and genetic variation to ascertain the importance of isolation by distance (up- versus down-river), as well as whether the Madeira serves as a barrier to gene flow for these mammals. We measured 45 morphological characters from preserved museum specimens and analyzed the data via principal components analysis and discriminant analysis. Genetic data were derived from sequences of the mitochondrial cytochrome *b* gene, which we analyzed via F-statistics and network construction. We found strong genetic differentiation between populations on different banks of the river ($F_{ST} = 0.62832$, $p<0.0001$), but morphological differentiation was comparatively weak; there was no evidence of isolation by distance on either side of the river. Our study provides compelling evidence that the Madeira and other large rivers can serve as barriers to gene flow, at least among small mammals.

Introduction

The aim of this study was to investigate the phylogenetic impact of the Madeira River on small mammals in the Amazon basin, using *Marmosa (Micoureus) demerarae* as a study species. By looking at populations of *M. demerarae* up and down as well as across the Madeira River, we hoped to shed light on the importance of the river in population divergence and, ultimately, speciation.

The Amazon

The Amazon basin covers 6.5 million square kilometers, comprising the largest and most species-rich tract of tropical rainforest in the world (Bermingham 2005). It extends into 9 countries, from roughly 10° north latitude to 15º south latitude; and the equatorial climate is permanently hot and humid with annual precipitation ranging from 1200 to 3000 millimeters. At present the area is largely unpopulated, but increased urbanization and forest fragmentation make the Amazon rainforest a subject of high conservation concern (de Miranda and Mattos 1992).

The Amazon River has over 1,100 tributaries including the Madeira and Rio Negro, which are each over 2,250 kilometers long. The Madeira River is the largest of these, flowing 3,380 km before joining the Amazon proper. As with all Amazonian rivers, its width can vary widely with the seasons, but it generally falls no lower than 1 km across (Sterling 1996). Alfred Russel Wallace, the "father of biogeography," spent several years studying Amazonian organismal diversity and hypothesized that the Amazon, Madeira, and Rio Negro function as species limits for small mammals (Figure 1):

FIGURE 1. Map of the Amazon as described by Wallace. The Amazon River, Madeira River, and Rio Negro serve as “limits beyond which certain species never pass” (1852).

"During my residence in the Amazon district I took every opportunity of determining the limits of species, and I soon found that the Amazon, the Rio Negro and the Madeira formed the limits beyond which certain species never passed. The native hunters are perfectly acquainted with this fact, and always cross over the river when they want to procure particular animals, which are found even on the river's bank on one side, but never by any chance on the other [sic]...Several Ecuador species from the east of the Andes reach down into the tongue of land between the Rio Negro and Upper Amazon, but pass neither of those rivers, and others from Peru are bounded on the north by the Upper Amazon, and on the east by the Madeira." (1852)

Marmosa (Micoureus) demerarae

Marsupials (metatherians) make up one of the major subcategories of the class Mammalia, distinguishable from their eutherian counterparts (placental mammals) on the basis of their young, which are born relatively underdeveloped. While many marsupials bear the archetypal "pouch," not all species have this feature (Armati et al. 2006). During the Cenozoic era, they were a major component of the South American land fauna with 16 families and 95 genera, but many clades have since become extinct (Marshall and Cifelli 1990). Currently 270 marsupial species have been identified worldwide, with 69 of those species occurring in South America (Armati et al. 2006). They represent the third greatest contributor to New World mammalian biodiversity after rodents and bats (R. S. Voss, Lunde, and Simmons 2001).

The 69 South American species belong to three extant families: Didelphidae (opossums: 15 genera, 63 species; Gardner 1993), Caenolestidae (rat opossums; 3 genera, 5 species; Marshall 1980), and Microbiotheriidae (monito del monte: 1 genus, 1 species; Marshall 1982). The didel-

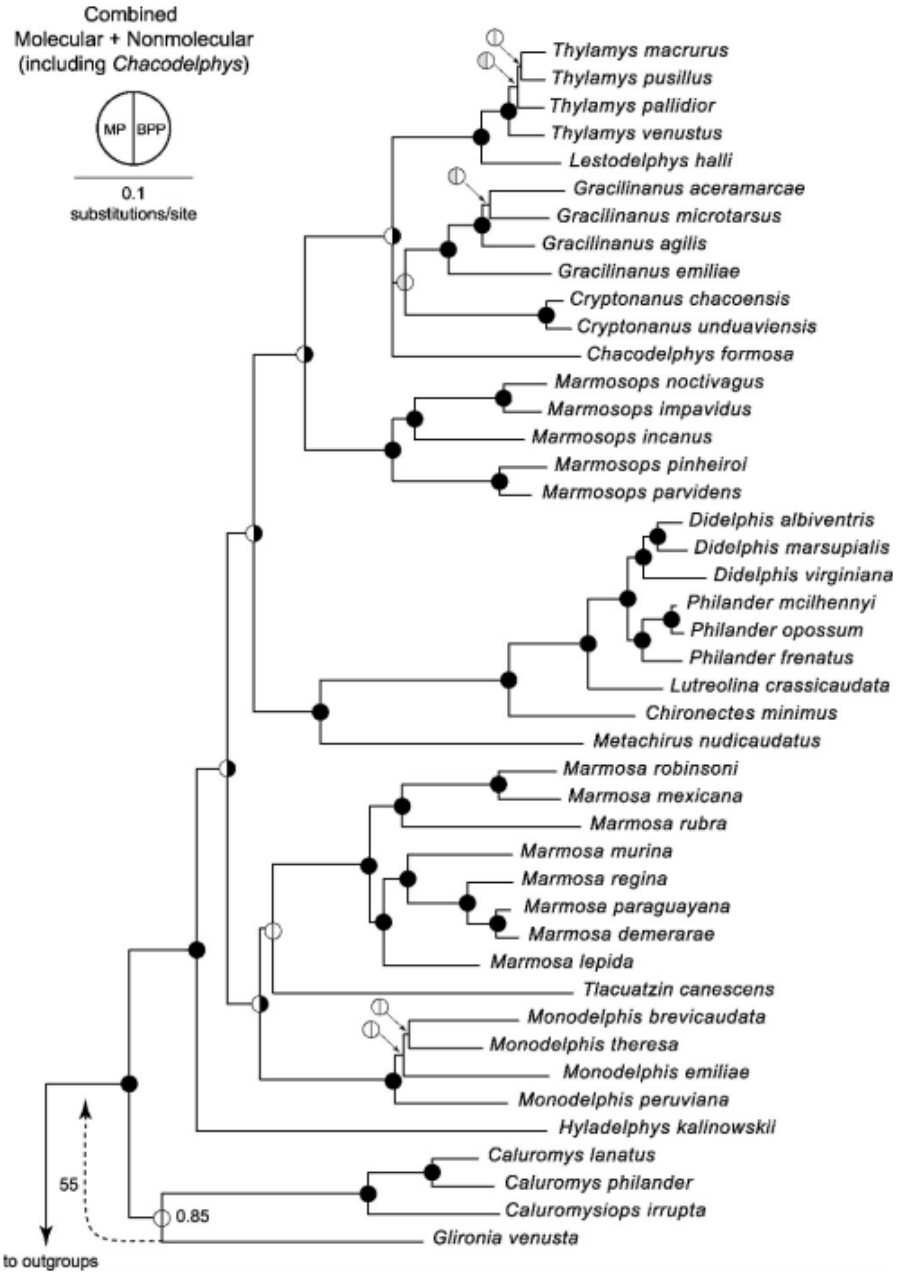


FIGURE 2. Current accepted Didelphid phylogeny (Voss and Jansa 2009). Support statistics from a parsimony bootstrap analysis (MP) and a Bayesian analysis (BPP) are indicated at each resolved node. For MP, white indicates bootstrap frequencies ≤50%, grey indicates bootstrap frequences between 50% and 75%, and black indicates bootstrap frequences ≥75%. For BPP, white indicates posterior probabilities <0.95, whereas black indicates posterior probabilities >0.95.

phids, by far the largest group, originated during the late Cretaceous in North America but radiated mainly during the early Cenozoic in South America (Szalay 1994; Figure 2). The family Didelphidae includes the species used in this study, the woolly mouse opossum *Marmosa (Micoureus) demerarae*, known locally as *cuíca*. This species was, until recently, part of the genus *Micoureus*, but was moved into *Marmosa* to correct for paraphyly with the name *Micoureus* retained as a subgenus (Voss and Jansa 2009).

M. demerarae is nocturnal, tree-dwelling, and largely solitary. It is highly mobile and has an insectivorous-omnivorous diet. Adults reach a weight of around 130 grams with some sexual dimorphism, females being slightly smaller than males (Gardner 1993; Figure 3). Diagnostic features include a dark circumocular mask, unpatterned dorsal pelage, and a tail that is substantially longer than the combined length of the head and body (Voss and Jansa 2009). Females lack a pouch; instead the young cling to their mother's teats or are left in nests while the mother feeds (Armati et al. 2006). *M. demerarae* ranges throughout the Amazon basin, from northern Colombia to northern Argentina, and east across Brazil to the Atlantic Ocean (Figure 4). It does not generally occupy high-elevation areas (Gardner 1993).

Recent genetic studies have identified highly-divergent phylogroups across this range, suggesting that *M. demerarae* may represent several species or subspecies. Carvalho et al. (2002) found gross banding differences in the karyotypes of *M. demerarae* individuals from the Cerrado and Mata Atlântica biomes, which they regarded as preliminary evidence suggesting the populations are in fact distinct species. Gutiérrez et al. (2010) also found mtDNA sequence differences of almost 6% between French Guianan and Peruvian *M. demerarae* populations and recommended that future studies examine the taxonomic importance of those data.

FIGURE 3. Three taxidermied *M. demerarae* individuals from the LEA - ESALQ small mammal collection. Right to left: male, female, juvenile.

FIGURE 4. South America; range of *M. demerarae* is shaded.

Morphological and Molecular Analyses

Since the early years of biological research, morphometrics, or the quantitative analysis of organismal forms, has served as the primary tool for identifying or distinguishing between species. Comparative anatomical research on vertebrates generally involves the use of both osteological and nonosteological data. In recent years, however, molecular analyses of evolutionary relationships have quickly gained in popularity, serving as a supplement or replacement to morphological analyses due to the powerful data they yield from relatively small sample sizes. Mitochondrial DNA (mtDNA) has become a favorite tool for genetic analysis in both inter- and intra-specific evolutionary studies (Avise 1994). The high rate of evolution and high copy number of sequences allow mtDNA to be readily amplified in the Polymerase Chain Reaction (PCR) and make it useful for building trees below the species level. Cytochrome *b* (cyt-b), which is involved in the oxidative phosphorylation system, is one of the more frequently utilized genes in the mitochondrial genome for these kinds of studies (Irwin et al. 1991).

Methods

63 individuals were collected from six localities in Sherman traps along a 60 kilometer stretch of the Madeira River near the Brazilian-Bolivian border (Figure 5). Collections were made during February, March, April, July, August and October 2010, and January, February, April, May and July of 2011. The six sites were Abunã-Left (AL; 9°37'S, 65°26'W), Abuna-Right (AR; 9°36'S, 65°21'W), Mutum-Left (ML; 9°35'S, 65°03'W), Mutum-Right (MR; 9°36'S, 65°02'W), Caiçara-Left (CL; 9°26'S, 64°50'W), and Caiçara-Right (CR; 9°26'S, 64°48'W). Specimens were then taxidermied and the skins, skulls, and liver tissue samples for each individual were stored at the ESALQ Animal Ecology Laboratory (LEA) or the Universidade de São Paulo mammal collection.

Morphological Analyses

Each of the 63 *M. demerarae* individuals was sexed and classified as juvenile or adult, with adults possessing a complete set of teeth (five incisors, one canine, three premolars, and four molars). Body weight and external body measurements (total length, tail length, body length, ear length, hindfoot length, and hindfoot length with claws) were recorded to the nearest millimeter in the field. Only those preserved specimens which were then stored in the LEA mammal collection (40 skulls and 40 skins) were used for the remaining morphological analyses (Table 1).

23 cranial measurements were taken with digital calipers from 40 preserved skulls as follows: greatest skull length (GSL), zygomatic breadth (ZB), breadth of braincase (Bbra), least interorbital breadth (LIB), least postorbital breadth (LPB), nasal length (NL), breadth across bullae (Bbul), palatal length (PL), palatal breadth (PB), maxillary toothrow (MTR), molar length (LM),

FIGURE 5. Map of the collection sites used in this study. The solid black box (top) is zoomed in (bottom) to show the names and locations of specific sites and river margins.

TABLE 1. All individuals used in this study. Field data was obtained for all 63 specimens, while skull, skin and molecular (tissue) data was only collected for those specimens that were stored in the LEA - ESALQ small mammal collection. Total number of individuals in each category are listed at the bottom.

ID	Field	Skull	Skin	Tissue	ID	Field	Skull	Skin	Tissue	ID	Field	Skull	Skin	Tissue
MJ1	X	X	X	X	MJ82	X				MJ297	X	X	X	X
MJ3	X	X	X	X	MJ84	X			X	MJ309	X	X	X	X
MJ5	X	X	X		MJ87	X				MJ310	X	X	X	X
MJ9	X	X	X	X	MJ88	X				MJ311	X	X	X	
MJ10	X	X	X		MJ89	X				MJ314	X	X	X	
MJ22	X	X	X	X	MJ93	X				MJ315	X	X	X	
MJ24	X	X	X		MJ94	X			X	MJ326	X	X	X	
MJ25	X	X	X	X	MJ96	X				MJ327	X	X	X	X
MJ28	X	X	X		MJ97	X				MJ334	X			
MJ29	X	X	X		MJ98	X				MJ341	X	X	X	
MJ30	X	X	X		MJ103	X			X	MJ342	X	X	X	
MJ48	X				MJ105	X		X		MJ 344	X	X	X	X
MJ49	X				MJ195	X	X	X	X	MJ 348	X	X	X	X
MJ51	X				MJ212	X	X	X		MJ 349	X	X	X	
MJ52	X				MJ231	X	X	X		MJ 351	X	X		
MJ53	X				MJ239	X	X	X		MJ 355	X	X	X	
MJ56	X				MJ249	X	X	X	X	MJ 356	X	X	X	
MJ58	X			X	MJ250	X	X	X		MJ 357	X	X	X	X
MJ66	X				MJ265	X	X	X	X	MJ 369	X	X	X	X
MJ68	X				MJ292	X	X	X		MJ 375	X	X	X	
MJ79	X				MJ294	X	X	X	X	MJ 379	X	X	X	X

NUMBER OF INDIVIDUALS WITH FIELD DATA: 63 SKULL DATA: 40 SKIN DATA: 40 MOLECULAR DATA: 22

breadth of rostrum (BR), cranial depth (CD), width of cranial opening (LCO), length of cranial opening to basioccipital (CB), Cranio-rostral length (CR), nasal bone width (LN), height of zygomatic flare (HZ), greatest interorbital width (MSW), length of first molar (M1), length of second molar (M2), length of third molar (M3), and length of fourth molar (M4). 19 measurements are shown in Figure 6.

Ordinal data was collected from skulls and skins for additional traits: coat color, tail color, ventral fur pattern, darkness of eyespots, color of ventral fur, prominence of sagittal ridges, prominence of sagittal crest, prominence of vertebral condyles, shape of zygomatic arch, shape of mandibular shelf, and shape of interorbital shelf. Multivariate analyses and univariate T-tests of morphological traits were run on the JMP 9 statistical platform (SAS Institute Inc. 2009)

Molecular Analyses

685 base pairs (bp) of the mitochondrial cyt-b gene were sequenced for 22 *M. demerarae* individuals (Table 2). Four of these 22 individuals were collected from Caiçara-Right, 3 from Caiçara-Left, 4 from Abunã-Right, 6 from Abunã-Left, 2 from Mutum-Right, and 3 from Mutum-Left. The sequences of three additional individuals from the genus *Marmosa—M. regina*, *M. murina*, and *M. paraguayana*—were obtained from Gutierrez et al. (2010; GenBank accession numbers HM106370.1, HM106395.1, and HM106372.1, respectively).

DNA was extracted from ethanol-preserved liver tissues using the ChargeSwitch ® gDNA Mini Tissue Kit. Cyt-b was amplified via PCR (Saiki and et al. 1988) using primer pairs MVZ05 and MVZ16. Amplifications were performed with a programmable thermocycler in 45 cycles with denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for

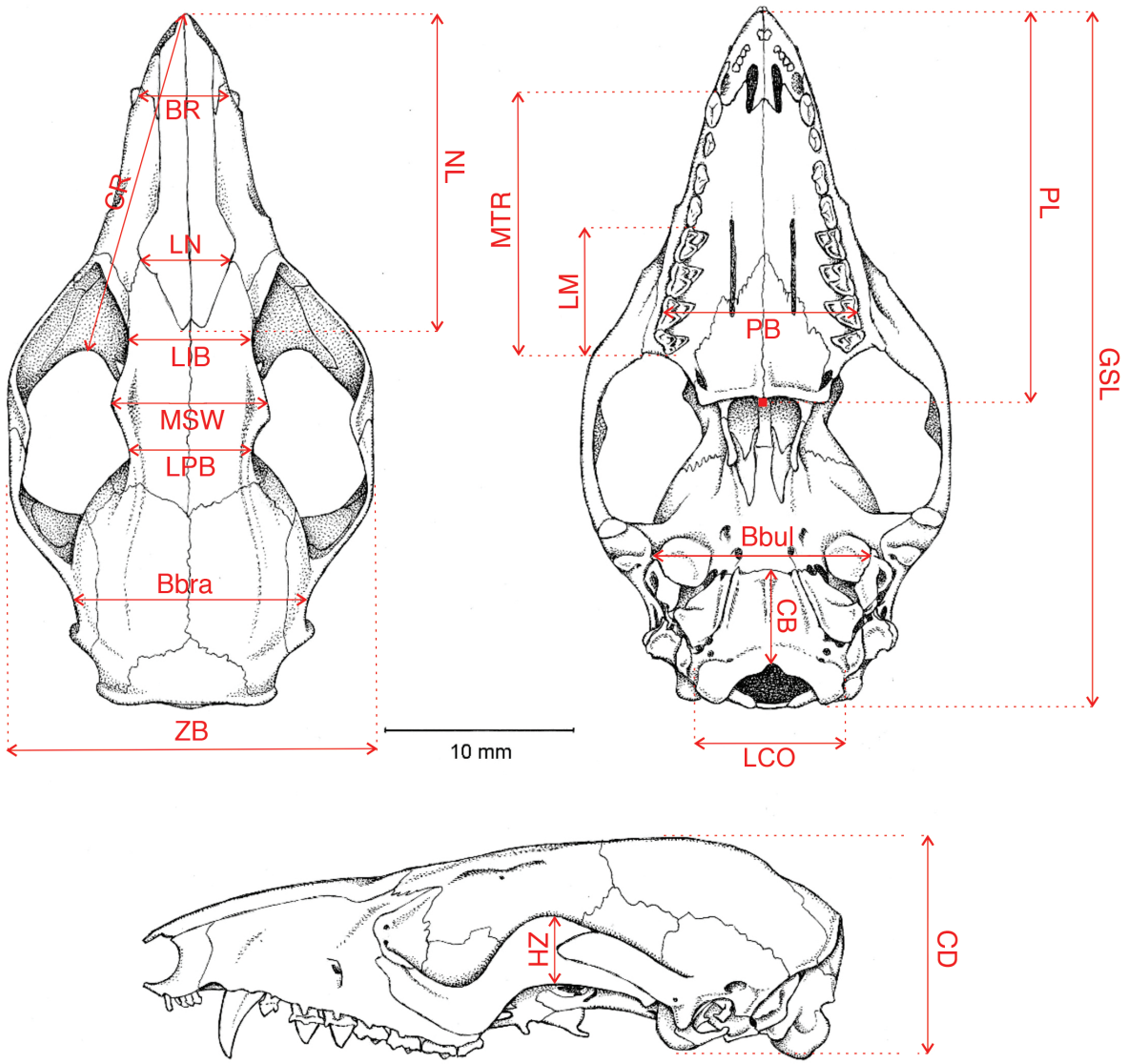


FIGURE 6. 19 cranial measurements recorded from 40 *M. demerarae* skulls, as follows: greatest skull length (GSL), zygomatic breadth (ZB), breadth of braincase (Bbra), least interorbital breadth (LIB), least postorbital breadth (LPB), nasal length (NL), breadth across bullae (Bbul), palatal length (PL), palatal breadth (PB), maxillary toothrow (MTR), molar length (LM), breadth of rostrum (BR), cranial depth (CD), width of cranial opening (LCO), length of cranial opening to basioccipital (CB), Cranio-rostral length (CR), nasal bone width (LN), height of zygomatic flare (HZ), and greatest interorbital width (MSW)

one minute 30 seconds. Samples were then sent to the Center for Molecular Biology and Genetic Engineering at the State University of Campinas in São Paulo to be cleaned and sequenced.

All sequences were aligned in the program BioEdit, which was also used to identify haplotypes and polymorphic sites. Estimates of pairwise sequence divergence values were generated by the Molecular Evolutionary Genetics Analysis (MEGA) program version 5.0 (Tamura et al. 2011), corrected by the Tamura 3-parameter model (Tamura 1992). F-statistics were calculated from DNA sequences in the program Arlequin 3.5 (Excoffier and Lischer 2010).

A phylogenetic analysis was performed via the neighbor-joining method (Saitou and Nei 1987). Tree construction and associated bootstrap analyses were performed in the program MEGA with 1000 replications. The tree was drawn with branch lengths proportional to the number of nucleotide changes along each. A population network was also constructed from DNA sequences by the program TCS version 1.21, which incorporated haplotype frequency in its output (Clement et al. 2000).

TABLE 2. List of individuals used in molecular analyses. Age is given as adult (A) or juvenile (J). Sex is male (M) or female (F). Site is the locality from which the individual was collected, given as Caiçara (C), Mutum (M), or Abunã (A). Margin is the river margin from which the individual was collected, given as right (R) or left (L).

ID	Age	Sex	Site	Margin
MJ1	A	M	C	R
MJ3	A	M	C	L
MJ9	A	M	C	R
MJ22	A	F	C	L
MJ25	J	F	M	R
MJ58	A	M	A	R
MJ84	A	F	A	L
MJ94	A	M	A	L
MJ103	A	F	A	L
MJ195	A	M	A	R
MJ249	A	M	A	R
MJ265	A	F	M	R
MJ294	J	M	C	R
MJ297	A	F	C	L
MJ309	J	F	A	L
MJ310	A	M	A	L
MJ327	J	F	M	L
MJ 344	A	F	A	R
MJ 348	A	F	A	L
MJ 357	A	M	M	L
MJ 369	A	M	M	L
MJ 379	A	F	C	R

Results

Morphological

Multivariate analyses of morphological traits failed to uncover significant evidence of differentiation among populations. Principal components analysis accounted for >70% of the variation in three factors (Figure 7, Table 3), but those factors did not separate right- and left-margin populations in multivariate space (Figure 8). Discriminant analysis was unable to accurately predict population based on morphological traits, misclassifying individuals 20% of the time for adult females and 53.33% of the time for adult males (Table 4).

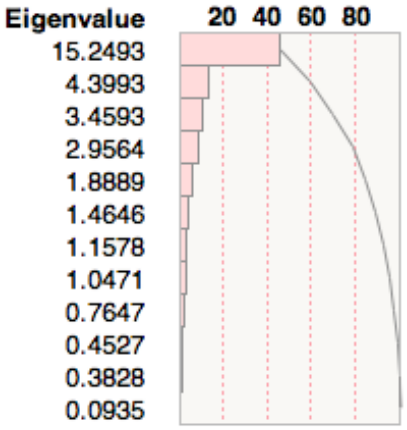
Univariate T-tests hinted at incipient morphological separation, yielding statistically significant differences for 3 of the 30 quantitative traits between right and left sides of the river (hindfoot length, hindfoot length with claws, and craniorostral length), but these were rendered insignifict after a Bonferroni correction (Table 5; Bonferroni 1935).

Molecular

A total of 12 distinct haplotypes were recovered among the 22 individuals of *M. demerarae* sampled, and 35 sites of the 685 bp examined in the mitochondrial cyt-b gene were found to be polymorphic (Table 6). A matrix of Tamura 3-parameter distances among all individuals is given in Table 7 (Tamura 1992). Levels of sequence divergence range from 0.0% (identical haplotypes) to 3.8% across our samples.

Pairwise F-Statistics between sites on the same river margins showed no significant populational differentiation (Table 8; on the right, mean $F_{ST} = 0.3616$, $P > 0.05$; on the left, mean $F_{ST} =$

(A) Adult females



(B) Adult males.

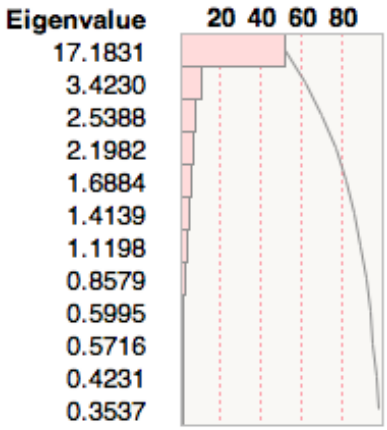


FIGURE 7. Principal Components Analysis. Graphs show the first 12 principal components, their associated eigenvalues, and the relative contributions of each to the total variation.

TABLE 3. Principal Components Analysis loading matrices. Values and shading represent the contributions of each variable to Principal Components 1-3.

(A) Adult females.

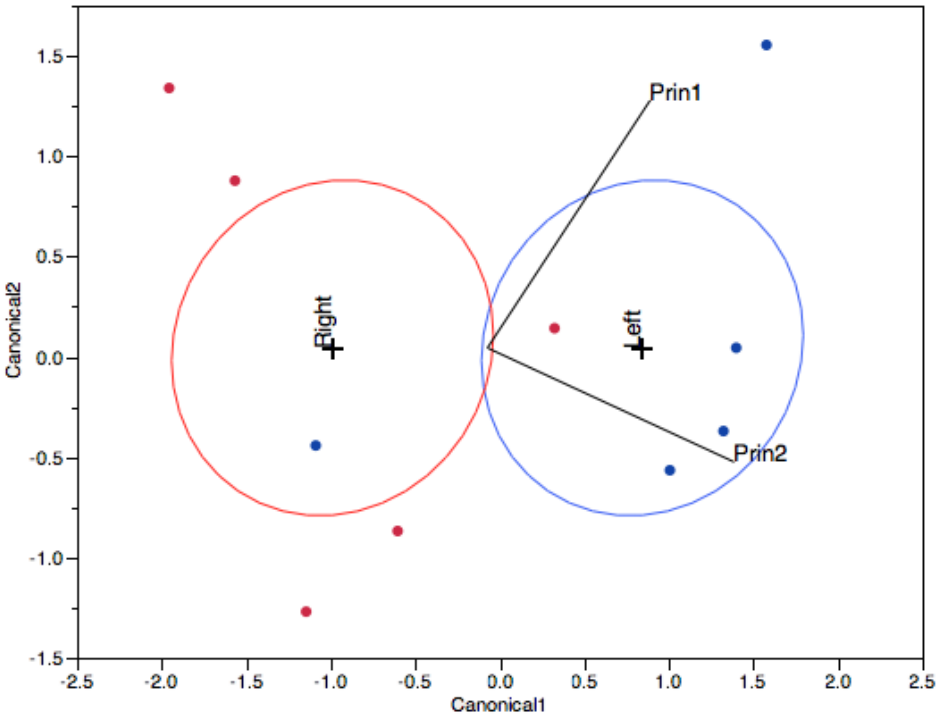
	Prin1	Prin2	Prin3
Total Length (cm)	0.55432	-0.34351	0.20038
Body Length (-tail)	0.76432	-0.46381	0.32940
Tail Length	0.83744	-0.00271	0.27476
Foot Length w/Claws	0.31569	0.47821	0.63934
Foot Length w/o claws	0.15573	0.36923	0.70765
Ear Length	0.49898	0.01827	0.63871
Weight (g)	0.90660	-0.29544	0.01139
Greatest Skull Length (GSL)	0.90486	0.18950	-0.05162
Zygomatic Breadth (ZB)	0.89738	-0.15805	-0.32613
Breadth of Braincase (Bbra)	0.88337	0.06187	-0.27522
Least Interorbital Breadth (LIB)	0.63445	-0.23423	-0.26769
Least Postorbital Breadth (LPB)	-0.75238	-0.36922	-0.01443
Nasal Length (NL)	0.72769	0.30361	0.07794
Breadth across Bullae (Bbul)	0.34630	0.82132	-0.18554
Palatal Length (PL)	0.91722	0.23849	-0.16401
Palatal Breadth (PB)	0.86293	-0.01627	0.28540
Maxillary Toothrow (MTR)	0.78019	0.25331	0.24701
Molar Length (LM)	-0.17573	0.68975	0.13818
Breadth of Rostrum (BR)	0.89420	-0.26871	0.06895
Cranial Depth (CD)	0.48198	0.11048	0.11956
LCO	-0.05430	0.89511	-0.34793
CB	0.91715	-0.08670	-0.27760
CR	0.66670	0.47094	0.10313
LN	0.59606	0.11591	0.34661
Height of Zygomatic Flare	0.74095	-0.59633	-0.08621
Intraorbital Shelf Width (cm)	0.69184	-0.43397	-0.27418
Length of Furry Part of Tail	0.69263	0.12431	-0.16343
M1	-0.63401	-0.04537	0.46995
M2	-0.47456	-0.20285	0.56926
M3	-0.35294	-0.28013	0.43716
M4	0.43553	-0.24495	0.48749

(B) Adult males.

	Prin1	Prin2	Prin3
Total Length (cm)	0.82366	-0.10178	0.38259
Body Length (-tail)	0.72524	-0.20611	0.39214
Tail Length	0.67714	0.12259	0.21685
Foot Length w/Claws	0.27683	0.19013	0.85818
Foot Length w/o claws	0.50937	0.41498	0.69169
Ear Length	0.54567	0.15114	0.00638
Weight (g)	0.94538	-0.07290	-0.22556
Greatest Skull Length (GSL)	0.97446	0.08286	-0.14649
Zygomatic Breadth (ZB)	0.95491	-0.11424	-0.19224
Breadth of Braincase (Bbra)	0.97262	0.03273	-0.11017
Least Interorbital Breadth (LIB)	0.89716	-0.06262	-0.24040
Least Postorbital Breadth (LPB)	-0.11306	0.00912	0.04640
Nasal Length (NL)	0.93454	0.21318	-0.10730
Breadth across Bullae (Bbul)	0.85973	0.07869	0.16706
Palatal Length (PL)	0.96705	0.04738	-0.11882
Palatal Breadth (PB)	0.80585	0.16029	-0.29624
Maxillary Toothrow (MTR)	0.72223	0.53619	-0.22286
Molar Length (LM)	-0.12246	0.72660	-0.38744
Breadth of Rostrum (BR)	0.87488	-0.15027	-0.27699
Cranial Depth (CD)	0.90184	0.00149	0.07437
LCO	0.19621	0.43245	0.39196
CB	0.88554	-0.02384	-0.11154
CR	0.75773	0.27450	-0.01529
LN	0.78605	-0.27544	-0.01337
Height of Zygomatic Flare	0.81868	-0.42447	0.08937
Intraorbital Shelf Width (cm)	0.90084	-0.18609	-0.05093
Length of Furry Part of Tail	0.66090	0.13222	-0.09039
M1	-0.10539	0.64824	0.11102
M2	-0.13475	0.43404	0.19442
M3	-0.36586	0.56990	-0.23828
M4	0.13426	0.80752	-0.11103

FIGURE 8. Canonical Discriminant Analysis Plots. Red dots represent individuals from the right river margin, blue dots represent individuals from the left river margin.

(A) Adult females



(B) Adult males

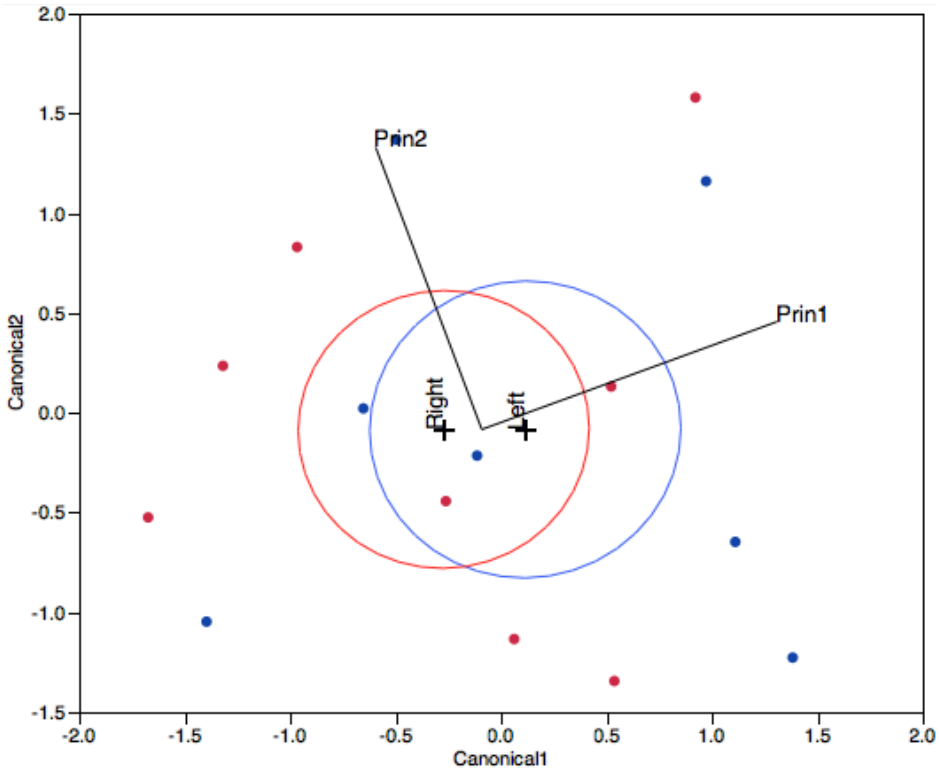


TABLE 4. Discriminant Analysis. Tables express the ability of these morphological data to discriminate effectively between the right- and left-margin populations.

(A) Adult females

		Predicted Group		
	Margin	Left	Right	Total
Count	Left	4	1	5
	Right	1	4	5
%	Left	80.0	20.0	100.0
	Right	20.0	80.0	100.0

Total Number Misclassified = 2; Total Percent Misclassified = 20%

-2loglikelihood = 7.866

(B) Adult males

		Predicted Group		
	Margin	Left	Right	Total
Count	Left	3	4	7
	Right	4	4	8
%	Left	42.9	57.1	100.0
	Right	50.0	50.0	100.0

Total Number Misclassified = 8; Total Percent Misclassified = 53.33%

-2loglikelihood = 20.17

-0.0544, P >0.05). This result indicates considerable gene flow up and down the river's edge. Conversely, genetic differentiation between populations on each river margin was strong and highly significant (F_{ST} = 0.62832, p<0.0001). That is, we found very little gene flow between populations across the river from each other.

A neighbor-joining phylogenetic diagram identified 2 distinct *M. demerarae* phylogroups (Figure 9). These groups were roughly defined by the river margins from which these individuals originated, with 2 outlying individuals (MJ195 and MJ58). Bootstrap values for these branches were >90. This tree supported the sister relationships between *M. demerarae*, *M. regina*, *M. murina*, and *M. paraguayana* that were previously defined by Voss and Jansa (2009)

A population network was also constructed to identify phylogenetic relationships among the sequenced *M. demerarae* individuals (Figure 10). These diagrams incorporated haplotype frequency, and identified 17 discrete single-nucleotide mutations separating the two major clades. When haplotypes on this diagram were labeled by river margins, two distinct phylogroups were identified.

TABLE 5. External and cranial measurements of *M. demerarae* individuals from the right and left river margins of the Madeira River[a]

	Right			Left	
Character	Male	Female		Male	Female
Total Length	409.0±40.23	401.1±39.69	ns (ns)	434.7±34.07	391.3±33.75
	N = 18	N = 14	ns (ns)	N = 12	N = 8
Body Length	170.2±22.45	173.1±26.18	ns (ns)	185.0±30.03	168.3±29.60
	N = 18	N = 14	ns (ns)	N = 12	N = 8
Tail Length	238.8±20.81	234.9±15.25	ns (ns)	249.7±12.70	223.0±37.03
	N = 18	N = 14	ns (ns)	N = 12	N = 8
Hindfoot Length + Claws	28.4±2.01	27.1±2.11	* (ns)	30.5±2.23	27.0±1.16
	N = 18	N = 14	ns (ns)	N = 12	N = 8
Hindfoot Length	26.8±1.83	25.3±1.90	* (ns)	28.9±2.11	25.1±1.58
	N = 15	N = 14	ns (ns)	N = 12	N = 8
Ear Length	28.0±2.95	27.5±2.17	ns (ns)	28.6±2.25	26.9±2.34
	N = 18	N = 14	ns (ns)	N = 11	N = 7
Weight (g)	121.7±37.26	94.1±17.55	ns (ns)	122.7±28.71	91.6±25.77
	N = 17	N = 14	ns (ns)	N = 12	N = 7
GSL	45.5±2.73	41.9±1.67	ns (ns)	44.9±2.46	43.2±2.25
	N = 10	N = 6	ns (ns)	N = 8	N = 6
ZB	25.6±1.95	24.1±1.34	ns (ns)	25.1±1.85	23.8±1.19
	N = 10	N = 6	ns (ns)	N = 8	N = 6
Bbra	16.1±1.04	14.9±0.52	ns (ns)	16.0±0.97	15.3±.75
	N = 10	N = 6	ns (ns)	N = 8	N = 6
LIB	8.2±0.97	7.4±0.36	ns (ns)	7.9±0.66	7.3±0.31
	N = 10	N = 6	ns (ns)	N = 8	N = 6
LPB	6.3±0.62	6.6±0.18	ns (ns)	6.6±0.21	6.2±0.51
	N = 10	N = 6	ns (ns)	N = 8	N = 6
NL	20.2±1.74	18.4±1.14	ns (ns)	20.1±1.11	19.6±1.39
	N = 10	N = 6	ns (ns)	N = 8	N = 6
Bbul	14.2±0.69	13.4±0.42	ns (ns)	14.3±0.78	13.6±0.47
	N = 10	N = 6	ns (ns)	N = 8	N = 6
PL	25.0±1.28	23.4±1.06	ns (ns)	25.0±1.27	23.8±1.00
	N = 10	N = 6	ns (ns)	N = 8	N = 6

PB	14.4±0.59 N = 10	13.9±0.41 N = 6	ns (ns) ns (ns)	14.2±0.73 N = 8	13.9±0.53 N = 6
MTR	17.9±0.50 N = 10	17.2±0.38 N = 6	ns (ns) ns (ns)	17.8±0.49 N = 8	17.4±0.56 N = 6
LM	9.1±0.26 N = 10	10.2±3.67 N = 6	ns (ns) ns (ns)	9.0±0.25 N = 8	8.9±0.10 N = 6
BR	9.0±0.73 N = 10	8.5±0.47 N = 6	ns (ns) ns (ns)	9.0±0.67 N = 8	8.6±0.57 N = 6
CD	11.3±0.48 N = 9	10.8±0.32 N = 6	ns (ns) ns (ns)	10.8±0.36 N = 8	11.3±0.23 N = 6
LCO	8.8±0.37 N = 9	8.3±0.35 N = 6	ns (ns) ns (ns)	9.0±0.32 N = 8	8.6±0.43 N = 6
CB	5.9±0.54 N = 9	5.3±0.49 N = 6	ns (ns) ns (ns)	5.8±0.50 N = 8	5.5±0.41 N = 6
CR	23.6±0.79 N = 10	22.0±0.43 N = 6	ns (ns) * (ns)	23.5±0.76 N = 8	22.7±0.49 N = 6
LN	6.3±0.60 N = 10	5.7±0.21 N = 6	ns (ns) ns (ns)	6.3±0.54 N = 8	5.8±0.68 N = 6
HZ	4.9±0.54 N = 9	4.6±0.50 N = 6	ns (ns) ns (ns)	5.0±0.53 N = 8	4.3±0.36 N = 6
MSW	9.6±1.39 N = 9	8.7±0.78 N = 6	ns (ns) ns (ns)	9.7±1.60 N = 8	8.2±0.65 N = 6
M1	2.2±0.18 N = 10	2.2±0.15 N = 6	ns (ns) ns (ns)	2.2±0.08 N = 8	2.2±0.15 N = 6
M2	2.4±0.12 N = 10	2.4±0.08 N = 6	ns (ns) ns (ns)	2.6±0.31 N = 8	2.5±0.23 N = 6
M3	2.9±0.25 N = 10	2.8±0.22 N = 6	ns (ns) ns (ns)	2.8±0.16 N = 8	2.8±0.20 N = 6
M4	3.1±0.11 N = 10	3.1±0.08 N = 6	ns (ns) ns (ns)	3.1±0.21 N = 8	3.1±0.11 N = 6

[a] Mean and standard deviation, with sample sizes below, for adult specimens of both sexes. The significance levels between the two species for both males (above) and females (below) are indicated: ns = $p>0.05$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. Significance after Bonferroni correction given in parentheses next to p-values.

TABLE 6. All polymorphic sites for each of the 12 unique *M. demerarae* haplotypes identified in this study. Header row gives the nucleotide position. Dots represent base pairs that are identical to those in haplotype 1. The last two columns indicate the haplotypes that were found on each river margin.

	22	28	107	172	178	190	193	205	208	211	220	253	256	271	284	295	310	325	406	424	433	442	460	502	527	529	541	-	565	583	595	640	641	642	643	646	Right Margin	Left Margin
Haplotype 1	C	A	G	C	T	T	A	C	C	A	T	T	T	A	G	C	A	A	T	A	G	C	T	T	C	A	G	-	T	T	T	G	G	C	C	C	1	6
Haplotype 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	-	.	.	.	.	.	.	.	.		2
Haplotype 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	-	.	.	.	.	.	.	.	.		1
Haplotype 4	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	-	.	.	.	.	.	.	.	.		2
Haplotype 5	.	.	.	T	.	.	.	.	T	.	.	.	.	G	.	T	.	.	.	.	.	.	.	.	.	.	A	-	G	.	.	.	.	.	.	.	1	
Haplotype 6	.	.	A	T	.	C	.	.	T	.	.	.	.	G	.	T	.	.	.	.	.	.	.	.	.	.	A	-	.	C	.	.	.	.	.	.		1
Haplotype 7	T	G	.	T	C	.	G	T	T	G	C	A	C	.	.	.	.	C	C	.	A	.	.	.	.	.	A	A	.	.	C	A	A	T	.	.	1	
Haplotype 8	T	G	.	T	C	.	G	T	T	G	C	A	C	.	.	.	G	C	C	.	A	.	.	.	T	.	A	-	.	.	C	A	A	T	.	.	2	
Haplotype 9	T	G	.	T	C	.	.	T	T	G	C	C	C	.	.	.	.	C	C	G	A	.	C	C	.	.	A	-	.	.	C	A	.	T	.	T	1	
Haplotype 10	T	G	.	T	C	.	.	T	T	G	C	.	C	.	.	.	.	C	C	G	A	.	C	C	.	.	A	-	.	.	C	A	.	T	.	T	2	
Haplotype 11	T	G	.	T	C	.	.	T	T	G	C	C	C	.	.	.	.	C	C	G	A	.	C	C	.	.	A	-	.	.	C	A	.	T	T	T	1	
Haplotype 12	T	G	.	T	C	.	.	T	T	G	C	.	C	.	A	.	.	C	C	G	A	.	C	C	.	.	A	-	.	.	C	A	.	T	.	T	1	

	MJ01	MJ03	MJ09	MJ22	MJ25	MJ84	MJ103	MJ195	MJ249	MJ265	MJ294	MJ297	MJ309	MJ310	MJ327	MJ356	MJ58	MJ94	MJ344	MJ348	MJ369
MJ01																					
MJ03	**0.030**																				
MJ09	0.011	**0.033**																			
MJ22	0.025	0.005	0.028																		
MJ25	0.003	**0.033**	0.014	0.028																	
MJ84	**0.030**	0.000	**0.033**	0.005	**0.033**																
MJ103	**0.030**	0.000	**0.033**	0.005	**0.033**	0.000															
MJ195	**0.030**	0.000	**0.033**	0.005	**0.033**	0.000	0.000														
MJ249	0.011	**0.032**	0.002	0.027	0.014	**0.032**	**0.032**	**0.032**													
MJ265	0.003	**0.033**	0.014	0.028	0.000	**0.033**	**0.033**	**0.033**	0.014												
MJ294	0.012	**0.033**	0.003	0.028	0.016	**0.033**	**0.033**	**0.033**	0.002	0.016											
MJ297	**0.030**	0.000	**0.033**	0.005	**0.033**	0.000	0.000	0.000	**0.032**	**0.033**	**0.033**										
MJ309	**0.030**	0.000	**0.033**	0.005	**0.033**	0.000	0.000	0.000	**0.032**	**0.033**	**0.033**	0.000									
MJ310	**0.030**	0.000	**0.033**	0.005	**0.033**	0.000	0.000	0.000	**0.032**	**0.033**	**0.033**	0.000	0.000								
MJ327	**0.032**	0.002	**0.035**	0.006	**0.035**	0.002	0.002	0.002	**0.033**	**0.035**	**0.035**	0.002	0.002	0.002							
MJ357	**0.033**	0.012	**0.036**	0.008	**0.036**	0.012	0.012	0.012	**0.035**	**0.036**	**0.036**	0.012	0.012	0.012	0.014						
MJ58	**0.030**	0.009	**0.033**	0.005	**0.033**	0.009	0.009	0.009	**0.032**	**0.033**	**0.033**	0.009	0.009	0.009	0.011	0.006					
MJ94	**0.032**	0.002	**0.035**	0.006	**0.035**	0.002	0.002	0.002	**0.033**	**0.035**	**0.035**	0.002	0.002	0.002	**0.003**	0.014	0.011				
MJ344	0.011	**0.032**	0.002	0.027	0.014	**0.032**	**0.032**	**0.032**	0.000	0.014	0.002	**0.032**	**0.032**	**0.032**	**0.033**	**0.035**	**0.032**	**0.033**			
MJ348	0.025	0.005	0.028	0.000	0.028	0.005	0.005	0.005	0.027	0.028	0.028	0.005	0.005	0.005	0.006	0.008	0.005	0.006	0.027		
MJ369	**0.032**	0.002	**0.035**	0.006	**0.035**	0.002	0.002	0.002	**0.033**	**0.035**	**0.035**	0.002	0.002	0.002	0.000	0.014	0.011	0.003	**0.033**	0.006	
MJ379	0.012	**0.035**	0.002	**0.030**	0.016	**0.035**	**0.035**	**0.035**	0.003	0.016	0.005	**0.035**	**0.035**	**0.035**	**0.036**	**0.038**	**0.035**	**0.036**	0.003	**0.030**	**0.036**

TABLE 7. Estimates of evolutionary divergence between sequences. Analyses were conducted using the Tamura 3-parameter model in MEGA 5. All positions containing gaps and missing data were eliminated. Sequence divergences range from 0.0% (identical haplotypes) to 3.8%. Sequence divergences ≥3% are in bold.

TABLE 8A. Population pairwise F_{ST} values[a]

	AR	CR	MR	AL	CL	ML
AR	0.00000					
CR	0.18440	0.00000				
MR	0.34226	0.55810	0.00000			
AL	0.40310	0.86865	0.94671	0.00000		
CL	0.19082	0.81399	0.93370	-0.21519	0.00000	
ML	0.18012	0.75936	0.82353	0.14286	-0.09091	0.00000

TABLE 8B. Population pairwise F_{ST} p-values

	AR	CR	MR	AL	CL	ML
AR	*					
CR	0.22523±0.0412	*				
MR	0.11712±0.0194	0.15315±0.0194	*			
AL	0.06306±0.0237	**0.00000±0.0000**	**0.04505±0.0244**	*		
CL	0.27027±0.0359	**0.04505±0.0203**	**0.04505±0.0203**	0.99099±0.0030	*	
ML	0.28829±0.0466	0.05405±0.0242	0.05405±0.0201	0.15315±0.0333	0.55856±0.0265	*

TABLE 8C. Population pairwise F_{ST} values and p-values for pooled right- and left-margins

Population 1	Population 2	F_{ST}	p-value
Right Margin (pooled)	Left Margin (pooled)	0.62832	**0.00000±0.0000**

[a] (A) Pairwise F_{ST} values between all six localities. Values above 0.20 with significant p-values are considered to have extremely high inter-populational genetic differentiation. (B) P-values for table 6A. Significant p-values in bold. 110 permutations. (C) Pairwise F_{ST} value and p-value between right and left river margins with all localities pooled. “Right Margin (pooled)” = AR + CR + MR; “Left Margin (pooled)” = AL + CL + ML.

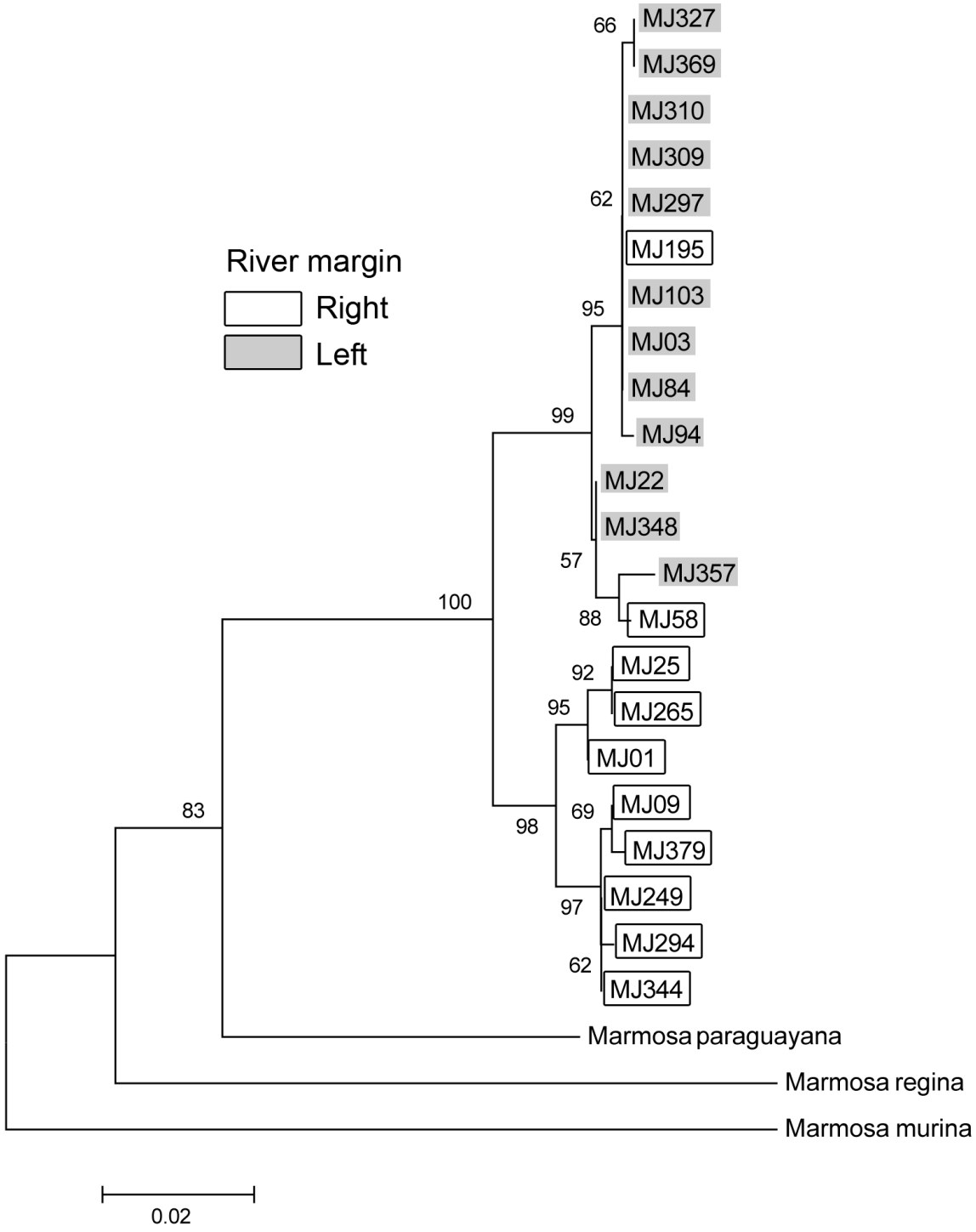


FIGURE 9. Neighbor-joining phylogeny for mtDNA sequence data based upon 1000 replicates. Bootstrap values at each branch. Outgroups: *Marmosa paraguayana* (HM106372.1), *Marmosa regina* (HM106370.1), and *Marmosa murina* (HM106395.1).

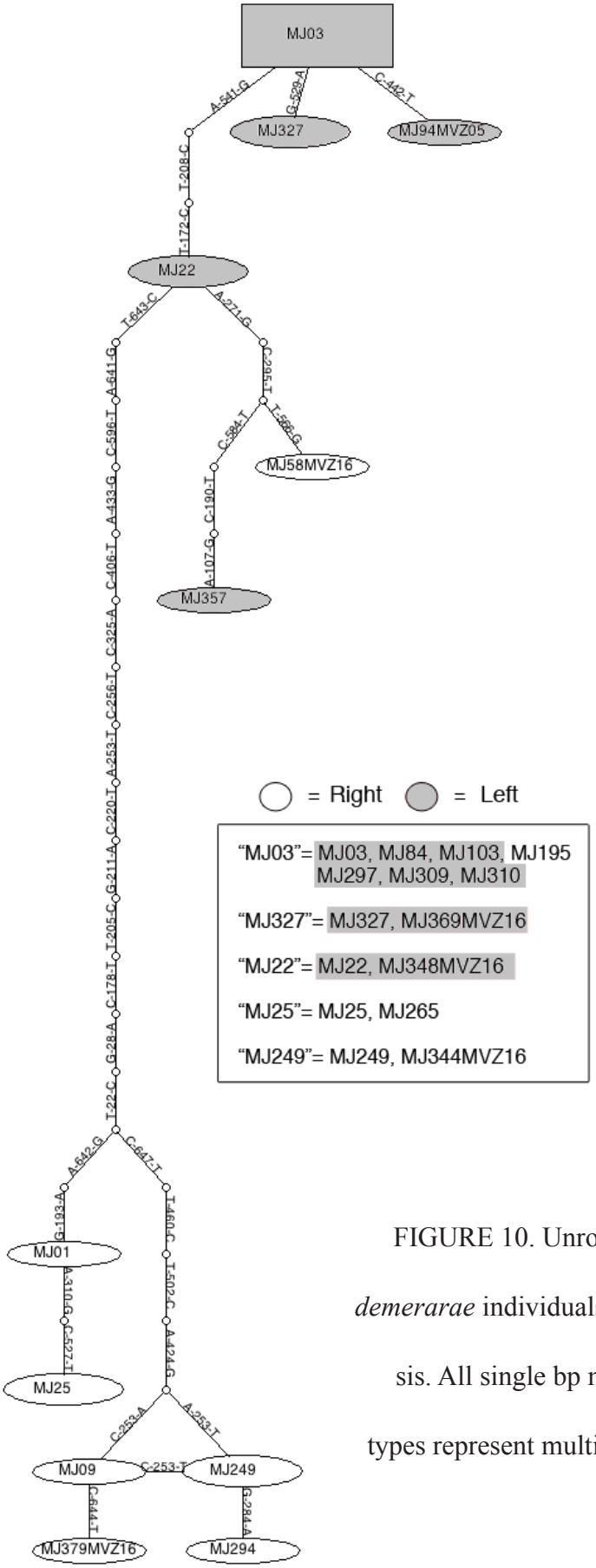


FIGURE 10. Unrooted population network of all *M. demerarae* individuals that underwent molecular analysis. All single bp mutations are shown. Some haplotypes represent multiple individuals, listed on the side.

Discussion

We found strong support for our hypothesis that population divergence in *M. demerarae* results from allopatric separation by the Madeira River. The low pairwise F_{ST} values and insignificant p-values for subpopulations along each river margin (AR/MR/CR and AL/ML/CL) suggest relatively high levels of gene flow between localities on the same side of the river. This is expected for *M. demerarae*, which is known to be a highly mobile animal (Gardner 1993). However, the incredibly large F_{ST} value of 0.62832 ($p<0.0001$) for populations across the river is indicative of highly restricted levels of gene flow between the right and left banks. Sequence divergence estimates between individuals on opposite river margins also reached a substantial 3.8%. Finally, phylogenetic analyses (Figures 9 and 10) also clearly suggest that the river has played a role in the divergence of these populations. In each diagram two distinct phylogroups were formed with high bootstrap values (>90) for support, separated clearly by river margin.

While our hypothesis was strongly supported by genetic data, we did not find similarly strong evidence from morphology. Principal Components Analysis was able to account for >70% of all morphological characters with three variables, but the right- and left-margin populations did not separate with any significance in multivariate space. Discriminant Analysis was unable to find any characters which could discriminate between the two populations with confidence. The univariate T-Test (Table 5) was the only statistical test of morphology to hint at incipient interpopulational differentiation, but that, too, was not significant after correction.

We attribute the negative results of morphological analysis to problems with the data structure. Firstly, sample sizes were fairly low, as we only had access to those specimens which were

being stored at the LEA-ESALQ small mammal collection. Low sample sizes for statistical analyses were further confounded by the different numbers of males, females, and juveniles across our study sites. Juveniles were not used in any morphological analyses, as their ages ranged from infant to young adult and could therefore not be compared to one another. Males and females could also not be compared to one another, as *M. demerarae* exhibits significant levels of sexual dimorphism. Therefore, some sample sizes were as low as n = 5 for one adult sex group on one river margin, drastically limiting statistical power. Genetic data were not plagued by the same problems because males, females, and juveniles could all be compared to one another and sample sizes were therefore comparatively large.

Imperfect population divergence in the form of two anomalous individuals (MJ58 and MJ195) whose molecular data suggests they originated on the opposite river margin could have several explanations unrelated to methodology. The presence of these individuals suggest that limited gene flow across the river may have occurred, but that mechanism is presently unclear. As the Madeira rarely falls below 1 kilometer across, it is unlikely that these individuals were able to swim across the river or jump from overhanging trees. It is also unlikely that these individuals traveled around the river to reach the opposite side, as the headwaters of the Madeira are over 1,000 kilometers southwest in the Peruvian Andes and outside the typical range of *M. demerarae*. Rafting is a distinct possibility here, as trees (which may play host to *M. demerarae* individuals) are often dislodged during rainy season floods and could be used to transport these marsupials. Our collection site was also approximately 50 kilometers upriver from the town of Porto Velho ("Old Port") which straddles the Madeira, so the anomalous individuals could have hitchhiked on one of the many boats that crosses the Madeira daily.

These two outlying individuals compounded by insignificant morphological results do not give much support to the presence of two distinct species on either river margin. At present there are no significant diagnostic characteristics by which the two clades can be confidently distinguished, and this study cannot rule out the possibility that they are capable of interbreeding. Additionally, in the genetic results, all *M. demerarae* individuals formed a well-defined clade that was much more separated from the three closely-related species than the two *M. demerarae* populations were separated from one another. Nonetheless, this study may serve as an early record of the origins of an allopatric speciation event.

Our study drew interesting conclusions about the function of rivers in evolution which may be extrapolated beyond the scope of marsupials across the Madeira River. We would imagine that other small animals or plants, provided they are not adept at water movement or transportation, would generate similar results in studies like this one. Additionally, we would expect to see similar results for rivers of comparable sizes such as the Rio Negro, Amazon and even the Mississippi, although the minimum river size necessary for gene flow inhibition is presently unknown.

The importance of our study could be far-reaching, not only in the field of phylogeography but also in conservation biology and present-day political issues. With increasing urbanization and development in Brazil, the results from our research and similar studies should be considered when developing policies. For example, Brazil is in the process of constructing several dams and bridges throughout the Amazon basin which could serve as corridors allowing non-native species to cross into previously uninhabited areas, potentially outcompeting their native counterparts (Tollefson 2012). More care could be taken to build these structures such that animals could not pass over them. Another example comes from a proposed law reform currently in Brazilian congress (as of

May 2012), which would allow more leniency to farmers wishing to mow down forest lands. Experts estimate this reform would contribute to the loss of 220,000 square kilometers of rainforest, mainly in the southeast region which is bound by the Amazon River to the north and the Madeira river to the West (Vidal and Carrington 2012). Our study provides additional documentation to support the idea that some species occupy just one of the four main Amazonian regions, which should be taken into account when considering policies that could severely deplete the habitat space of one region and drive down endemic population sizes.

Future endeavors to better understand the phylogeography of these and other Amazonian organisms should involve pointed fieldwork and a detailed comb through existing collections. The acquisition of new sources of phylogenetic data is another priority for future work in this field. Molecules that evolve more rapidly than cyt-b could contribute useful information about recent cladogenesis, and nontraditional morphological character systems like postcranial osteology and reproductive anatomy could also help expand this knowledge base. Finally, an identical or similar study to this one, conducted several years down the road, could provide informative data regarding the rate of population divergence and speciation across Amazonian rivers.

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